Cytotoxicity testing of biomaterials using methylcellulose cell culture. In vitro studies with in vivo verification using dermal sheep collagen

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- SUMMARY -

The general aim of this study was to investigate (crosslinked) dermal sheep collagen (DSC).
It concerns a fundamental *in vitro* study with *in vivo* verification in order to develop DSC's for clinical applications

Hexamethylenediisocyanate (HMDIC)-crosslinked DSC (HDSC) is commercially available as a wound dressing with the trade mark Tempocoll® (OPG Biomaterials BV, The Netherlands). Previous *in vitro* investigations with HDSC did not detect cytotoxic effects. However, it is known that isocyanates may induce cytotoxic effects, which may indicate that test methods used were not adequate.
This was the reason for the more specific aim of this study, i.e. the development of a *more sensitive in vitro cytotoxicity test system for biomaterials.*
The newly developed test system was based on a highly viscous methylcellulose gel and human fibroblasts were used as culture cells.
This *Methylcellulose (MC) cell culture* was found to be a universal test system, in which biomaterials can be tested in indirect and in direct contact with cells. Moreover, extracts of materials can be tested in this test system.

Advantages of MC cell culture, as compared to traditional test systems such as the agar-overlay test system and liquid test systems, which are accepted by the International Standard Organisation (ISO/TC 194), are:

1. Possibility of evaluating cytotoxicity with a growing cell line during a period of 7 days, without refreshing of the MC culture gel. This allows accumulation of possible cytotoxic products, i.e. detection of slow- or late-released cytotoxic products.
2. Cell counting is easily performed, resulting in clear quantification of the degree of cytotoxicity.
3. Cell morphology can be adequately examined by both light and transmission electron-microscopy.

Chapter 2 describes the new cytotoxicity test system and shows that the above mentioned HDSC induced a moderate cytotoxicity (± 50% inhibition of cell proliferation) with deviances in cell morphology, such as fatty degeneration.

Two types of cytotoxicity could clearly be discriminated with HDSC:

- *primary cytotoxicity*, which is due to leachables and
- *secondary* cytotoxicity, which is probably induced by cell-biomaterial interactions, (i.e. enzymatic actions.)

This observation was made from experiments with extracts and extracted counterparts of HDSC. All cytotoxic leachables were found to be extracted during 10 days, as observed by the *non*-cytotoxic second 10 day extract. However, *both* extracted counterparts, tested in the MC cell culture, continued to release cytotoxic products (±45% inhibition of cell proliferation). Therefore this secondary release most probably has to be related to enzymatic cleavage of the DSC. Enzymatic degradation may occur in the MC cell culture, since fibroblasts produce enzymes (e.g. collagenase), which may diffuse through the gel and there attack the biomaterial. Collagenase can specifically cleave the amino acid sequence leucine/glycine of the collagen molecule, thereby problably resulting in release of cytotoxic products.
The primary cytotoxicity is probably due to remnants of the crosslinking agent, i.e. HMDIC and/or its hydrolysis product 1,6-diaminohexane (DAH).

In Chapter 3, five groups of, mostly commercially available, wound dressings were tested in indirect contact with fibroblasts in MC cell culture, while Tempocoll® served as a reference material:

1. Conventional wound dressings,
2. Non-adhesive polyurethane wound dressings,
3. Adhesive polyurethane wound dressings,
4. Composites, and
5. Hydrocolloids

Only 5 out of 16 wound dressings did not induce cytotoxic effects. One group of wound dressings, i.e. the hydrocolloids, induced severe cytotoxic effects with inhibition of cell proliferation of more than 70%, and the remaining cells showed deviant morphologies.
The other wound dressings induced cell proliferation inhibition of ±15% to ±50%. With exception of the hydrocolloids, the degree of cytotoxicity was not clearly related to the type of the wound dressing. Some of these wound dressings, which were found to be cytotoxic in MC cell culture, previously were stated as non-cytotoxic according ISO/TC 194 standards. In our opinion, the ISO/TC 194 standards have to be re-defined, because cytotoxicity observed *in vitro* will influence wound healing and re-epithelialization *in vivo*. A highly cytotoxic wound dressing, such as a hydrocolloid, when applied *in vivo*, may cause, a high inflammatory reaction (hyperactivation) and this may be an explanation for the formation of hypergranulation

tissue as is often observed.
On the other hand, wound dressings, which in MC cell culture were found to be low or moderately cytotoxic, may be less harmful for reepithelialization if only used in case of contaminated wounds (potential antibacterial effect) or in case of a chronic wound reaction. With the latter, because cytotoxic products may activate wound healing reactions, e.g. influx of neutrophils and production of growth factors.

In Chapters 4, 5 and 6, detailed investigations on HDSC and two other commercially available DSC's, glutaraldehyde-crosslinked DSC (GDSC) and non-crosslinked DSC (NDSC), are reported.

In Chapter 4 the *in vitro* cytotoxicity of (crosslinked) DSC's using of serial extraction experiments is discussed.
HDSC and GDSC were serially extracted during 4x 10-day periods, and both the extracted DSC's and their respective extracts were tested in MC cell culture. NDSC was extracted during only one 10 day period, because of its sensitivity to aqueous hydrolysis.

GDSC, in contrast to HDSC, did not show a clear discrimination between primary and secondary cytotoxicity. The serial extracts of GDSC showed a continuing inhibition of cell proliferation of ±25% to ±40%, while the extracted GDSC had lost their structural integrity during 40 days of extraction. GDSC was found to be highly sensitive to aqueous hydrolysis.
Although the extractable cytotoxicity of GDSC was continuing up to 40 days, cytotoxicity of the extracted GDSC was strongly reduced (almost eliminated) after 2x 10 days of extraction. This shows that aqueous hydrolysis must be limited in the MC cell culture in comparison to the extraction procedure. Another indication is the low but continuing release of cytotoxicity from extracted GDSC's in the MC cell culture, which is the result from both hydrolytic and enzymatic actions (see Chapter 4).
The high sensitivity of GDSC to aqueous hydrolysis may be due to the presence of N=CH groups in Schiff's base-formed crosslinks. In contrast, HSDC was not found to be sensitive to aqueous hydrolysis during the extraction procedures.

With NDSC, only a low inhibition of cell proliferation without deviant cell morphology was found. This primary cytotoxicity is probably due to pre-crosslinking procedures of native DSC, such as pickling or pretreatment with proteolytic enzymes.

Chapter 5 addresses the question of whether the observed secondary cytotoxicity of HDSC (±40% inhibition of cell proliferation) can be eliminated by use of enzymatic pre-treatment, which might be important in case of *in vivo* applications of HDSC.
An indirect test method, as described below, is used, which may also show whether cell-biomaterial interactions can indeed occur in MC cell culture.
HDSC, purified from primary cytotoxic products, was incubated with enzyme-containing culture medium from a 2 day old liquid fibroblast culture. The results showed that enzyme-containing medium can once again release cytotoxic products from HDSC. However, in the MC

cell culture, the pretreated HDSC still induced inhibition of cell poliferation of ±25%, comparable to the control, which was HDSC pretreated with normal culture medium. Only after exposure for a *second* 6-day culture period to MC cell culture, pre-treated HDSC did no longer release secondary cytotoxic products (whereas the control still showed cytotoxicity). This proves that enzymatic interactions indeed occurred in MC cell culture and that secondary cytotoxicity can be eliminated.
The observed secondary cytotoxicity of HDSC may be due to pendant molecules in HDSC (see below). It is not likely that HMDIC-crosslinks (ureum-bonds) themselves are sensitive to aqueous hydrolysis or to enzymatic breakdown under culture conditions.

These results indicate that MC cell culture (in part) mimics the *in vivo* situation and can be used to detect cytotoxicity as a consequence of (i) leachable products, (ii) aqueous hydrolysis, and (iii) enzymatic actions. Exposure of biomaterials over a longer period of time to MC cell culture may therefore even more reflect the *in vivo* situation.

HDSC, GDSC and NDSC were, for this reason, repeatedly exposed to MC cell culture, during 6-day periods with a total of 42 days. From these results a detailed schematic presentation for the origin of cytotoxicity, also as a result of degradation, of HDSC, GDSC and NDSC, could be made (Chapter 6).

In case of HDSC, cytotoxicity was eliminated after 18 days of cell culture, but its structural integrity had not changed. This confirmed our hypothesis of pendant molecules being responsible for secondary cytotoxicity. Statistically, enzymatic attack in the collagen molecule will at first release fragments with pendant molecules, followed by release of fragments with 'intact' crosslinks. The latter products are probably still being released from HDSC from 18 up to 42 days, but obviously do not induce cytotoxicity.
Several types of pendant molecules may have been formed. One type of pendant molecule is based on a hydrolysis product of HMDIC, i.e. 1-amino-6 isocyanatehexane, which may have reacted with collagen. Another type of pendant molecule can be formed after reaction of HMDIC with another hydrolysis product, DAH, and with collagen.

In case of GDSC, the "bulk" of cytotoxicity was eliminated during 6 days MC cell culture, but a continuing low cytotoxicity was observed up to 42 days. As mentioned above, aqueous hydrolysis in MC cell culture is limited. Therefore, this "bulk" of cytotoxicity has to be related to remnants of GA present in GDSC. Thereafter, the continuing low cytotoxicity seems to be the result of two mechanisms, aqueous hydrolysis and enzymatic actions. As previously discussed a part of the GA-crosslinks is sensitive to aqueous hydrolysis. This may result in release of cytotoxic products and formation of new pendant molecules. Hydrolysis will decrease the structural integrity of GDSC, creating increased accessibility for enzymatic cleavage of collagen.The latter may further induce cytotoxicity by release of the (formed) pendant molecules. This explains the continuing low cytotoxicity of GDSC, which probably persists up to final degradation.

Fundamental *in vivo* studies on DSC's were carried out with subcutaneous implantation in rats to verify the *in vitro* results of DSC's. Detailed transmission electron-microscopical (TEM) evaluations of discs of GDSC, HDSC and NDSC were carried out during a period of 15 weeks.

With HDSC, a moderate cell infiltration with cell death and fatty degeneration, as well as a deviant morphology of neutrophils, was observed during a period of 14 days.
In case of GDSC, extreme cell death of infiltrated cells (mainly neutrophils) was observed up to 14 days. Moreover, high fatty degeneration of macrophages and giant cells was observed at least up to 3 weeks.
In case of NDSC only few neutrophils infiltrated. These were probably related to the surgical injury. Therefore it is concluded that NDSC did not elicit a cytotoxic reaction.
Furthermore, basophil-like blast cells were observed in each of the three types of DSC. These cells were able to form giant cells. Accumulation of aluminum silicate crystals, mostly present in elastin-like substances of DSC, was observed in the for basophils characteristic granula.
Fibroblast infiltration resulted in formation of new rat collagen in between remnants of only the *crosslinked* DSC's. Therefore, it was concluded that both DSC's could function as a temporary matrix. However, finally all collagenous parts were phagocytosed and intracellularly degraded by macrophage- and fibroblast- derived giant cells within 15 weeks. This indicates that a new collagen matrix will only remain where it meets functional demands.
With NDSC, macrophages and giant cells were not observed, which implies that phagocytosis will only occur with crosslinked collagen. NDSC is probably only extracellularly denatured. Fibroblasts were also not observed in NDSC. Therefore it is concluded that NDSC is not suitable as a temporary scaffold.

These results served as a reference to compare tissue interactions of "non-cytotoxic" DSC's, i.e. DSC's, which had been pretreated in tissue culture (TC-DSC's) (Chapter 7).
Discs of TC-HDSC, TC-GDSC and TC-NDSC (with inhibitions of cell proliferation of less than 10%), were implanted and evaluated over a period of 10 days by TEM.
TC-HDSC showed very low numbers of infiltrating neutrophils, without deviant cell morphology, which is, comparable to NDSC, probably related to the surgical injury.
With TC-GDSC, only a low infiltration of neutrophils and low fatty degeneration, but no extreme cell death, as observed with GDSC, were found. A large part of TC-GDSC had, in contrast to GDSC, not been infiltrated by fibroblasts and giant cells. This might be due to the denatured state of several collagen bundles of TC-GDSC and will as a consequence partly elicit tissue interactions comparable to those to NDSC.
TC-NDSC, as expected, did not show significant differences in cellular reactions as compared to NDSC.
The observed *in vivo* interactions with DSC's did correspond with the *in vitro* cytotoxicity results, obtained in MC cell culture.

In conclusion, we succeeded in developing a more sensitive in vitro cytotoxicity test system. MC cell culture was shown to function as a universal cytotoxicity test system for biomaterials, which can replace all cytotoxicity test systems required by the International Organization for Standardization. Furthermore it mimics, up to a certain limit, the *in vivo* situation. Future application of MC cell culture will result in more effective cytotoxicity scoring of candidate biomaterials and reduces the number of animals to be used for e.g. implantation tests.
The appendix of this thesis shows guidelines for using the MC cell culture.

The general aim of this study was to investigate (crosslinked) DSC's in order to develop DSC's for clinical applications. Both HDSC and GDSC were found to be cytotoxic, while NDSC only induced a very low cytotoxicity, without deviant cell morphology.
Washing will strongly reduce cytotoxicity of HDSC and GDSC and eliminate cytotoxicity of NDSC. However, washing should be carried out under controlled conditions in case of GDSC and NDSC, because of their sensitivity to aqueous hydrolysis. In case of application of rinsed GDSC we may expect a continuing low inflammatory reaction.

In general we may assume, that induction/regeneration of new tissue, i.e. the function as a temporary scaffold for generation of e.g. a neo-artery or neo-tendon, not only depends on the degree of cytotoxicity, but also depends on the presence of crosslinks in collagen-based materials. Therefore, we can conclude, that modifications of DSC with non-cytotoxic crosslinks (e.g. by carbodiimide) will be preferable for the future.
